CRYOPRESERVATION OF ENDOMETRIAL STROMAL CELLS OF BUFFALO (*BUBALUS BUBALIS*)

Chethan Sharma G., S.K. Singh*, Jessihun Nongsiej, H.B. Rakesh, R.P. Singh and S.K. Agarwal

ABSTRACT

The aim of the present experiment was to cryopreserve buffalo endometrial stromal cells and to study their morphological and physiological characteristics. Stromal cells isolated by enzymatic digestion from the buffalo uterus of day 1-5 of the estrous cycle, were cultured and frozen at -80°C using DMSO as a cryoprotectant. Frozen cells were thawed at 37°C and recultured. The culture media was collected when both unfrozen (control) and frozen cells reached confluence for estimation of PGE_2 and $PGF_{2\alpha}$ using enzyme-linked immunosorbent assay. Frozen stored buffalo endometrial stromal cells immediately after thawing appeared as single or in small clumps, glistening and rounded which changed their morphology from rounded to flat spindle shaped immediately 24 h after seeding retaining their normal morphological characteristics. The viability of frozen thawed stromal cells at the time of seeding estimated by Trypan blue dye exclusion was 63.3%. There was no significant difference in the basal production of PGE_2 and $PGF_{2\alpha}$ by the frozen-thawed and unfrozen buffalo stromal cells (289.38 ± 4.39, 3.28 ± 0.16 pg/µg protein vs. 305.97 ± 3.20, 4.26 ± 0.17 pg/µg protein, respectively) on day 7 of culture indicating no adverse effect of cryopreservation on their functional characteristics. The results demonstrated that buffalo endometrial stromal cells can be cryopreserved without any major impairment in their morphological and functional integrity.

Keywords: cryopreservation, buffalo, stromal cells, culture, prostaglandins

INTRODUCTION

Prostaglandins (PGE_2 & $PGF_{2\alpha}$) are important regulators of reproductive events viz., luteolysis, ovulation, implantation and parturition in farm animals. $PGF_{2\alpha}$ plays a major role in regulation of cyclicity, on the other hand, PGE_2 exerts action opposite to $PGF_{2\alpha}$ and favours establishment of pregnancy by its luteoprotective action. The endometrium is a complex tissue containing mainly epithelial and stromal cells. Both types of cells produce isoform of PG, but have different morphological and physiological properties (Fortier *et al.,* 1988; Asselin *et al.,* 1997). Endometrial epithelial cells mainly secrete $PGF_{2\alpha}$ whereas stromal cells are the principal source of PGE_2. Identification of a particular cell type responsible for observed effect and interaction between different cell types is difficult to study with mixed cell culture. An *in vitro* system for stromal cell culture, therefore, constitutes a good model to study regulation of prostaglandin synthesis (Parent *et al.,* 2003; Guzeloglu *et al.,* 2004), which avoids

Division of Animal Reproduction, Indian Veterinary Research Institute, Izatnagar, Bareilly (U.P.), 243 122, India *E-mail: singhsk2003@yahoo.co.in

animal handling, ethics problems and variability of results too. Recently, stromal cell culture (Gupta *et al.,* 2010) has also been established in buffalo in our laboratory, as a model to study the regulation and modulation of $PGF_{2α}$ and PGE_2.

To study the regulatory mechanism involved in PG synthesis related to either luteolysis or establishment of pregnancy, there is a need to cryopreserve uterine endometrial cells in order to conduct *in vitro* experiment at any point of time. Cryopreservation has most commonly been used for the long term storage of biological cells. Freezing and storage of endometrial cells allows accessibility of cells to conduct experiment at any point of time, reduces repeated procurement of genital tracts for isolation of cells and reduction in variability of results as well. Cryopreservation of cells derived from reproductive tracts e.g. bovine endometrial epithelial and stromal cells (Murakami *et al.,* 2003), bovine endothelial cells from the corpus luteum (Acosta *et al.,* 2007), porcine endometrial epithelial cells (Kim *et al.,* 2010) and equine endometrial epithelial and stromal cells (Szostek *et al.,* 2012) have been established. Till date, information on cryopreservation of stromal cells in buffalo is lacking. The present experiment, therefore, was designed with the objective to study the effect of cryopreservation on stromal cell morphology and physiology.

MATERIALS AND METHODS

Isolation of endometrial stromal cells

Buffalo uteri along with intact ovaries having corpus hemmorrhagicum (approximately day 1-5 of the estrous cycle) apparently free of infection were procured from the local abattoir, placed in sterile HBSS supplemented with antibiotics and transported to the laboratory on ice immediately after slaughter. White side test (Popov, 1969) and uterine cytology (Kasimanickam *et al.,* 2004) were done to rule out infection.

Buffalo endometrial stromal cells were isolated by enzymatic digestion as per Gupta *et al.* (2010) with suitable modifications. Briefly, the myometrial layers were dissected and the horn was everted to expose the uterine epithelium and placed in a beaker containing 0.3% trypsin III in HBSS and digested for 2 h at 5% CO_2, 95% humidified air at 37°C. After incubation the digested horn was further placed for second digestion containing 0.064% trypsin III and collagenase type II and 0.032% DNase I for 1 h. All scrapings and washings were combined with the digested cells and fetal calf serum was added to a final concentration of 10% to block the action of trypsin followed by centrifugation at 1200 rpm for 10 minutes. The pellets were pooled and washed thrice with HBSS and finally suspended in 1 ml RPMI-1640 medium with 10% FCS, 50 µg/ml gentamicin and 0.25 µg/ml Amphotericin-B. Cells were counted using haemocytometer and viability was determined by trypan blue dye exclusion test (Freshney, 2000). Cell concentration was adjusted to $6.25X10^5$ cells/ml in RPMI-1640 media plated on 6 well plates and cultured at 37°C in an atmosphere of 5% CO_2, 95% humidified air for 18 h to allow attachment of stromal cells. Media were changed every 2 days until monolayer. Further, stromal cells were subjected to indirect immuno-fluorescent staining for specific marker i.e. fibronectin (stromal cells). Cells stained positive giving green colored fluorescence indicating presence of specific stromal cells (Figure 1-D).

Cells were harvested upon reaching monolayer using 0.05% trypsin and 0.02% EDTA for 5-10 minutes at 37°C and washed two times with HBSS. Final cell pellet was suspended in chilled freezing media (70% culture media + 10% DMSO + 20% serum) to adjust cell concentration at the density of $1X10^6$ cells/ml after counting and viability assessment. One ml of cell suspension was dispensed into 2 ml cryovial and subjected to slow freezing in cryo 1°C freezing container (isopropanol bath) and kept at -80°C in a deep freezer. Cells were thawed by plunging cryovials into a water bath maintained at 37°C. Contents were centrifuged and the cell pellet was suspended in RPMI 1640 media with 10% FCS supplemented with antibiotics and counted using haemocytometer, the quantitative survivability of the cells was determined by staining the frozen thawed cells with 0.4%Trypan blue (Freshney, 2000). Growth characteristics of the frozen thawed stromal cells were evaluated by re-culturing the cells in RPMI-1640 at a density of $1X10^5$ viable cells/ml in 6 well plate. Media were changed every 2 days till confluence/ monolayer.

The supernatant were collected after reaching monolayer on day 7 for PGE_2 and $PGF_{2α}$ estimation using an ELISA Kit (Neogen, U.S.A). Cells were harvested using 0.05% trypsin and 0.02% EDTA for 5-10 minutes at 37°C. The cell pellets were dissolved with 0.3 M NaOH and 1% sodium lauryl sulfate and the protein concentration of cell lysates was determined by the Bradford method (Bradford, 1976) using BSA as standard (Banglore Genei, Banglore). Total PGE_2 and $PGF_{2α}$ concentration per well were expressed as picograms per microgram of total cellular protein.

Statistical analysis

The data are shown as mean ± SEM of values obtained in three separate experiments, each performed in triplicate. The independent ‘t’ test was used to show the statistical significance among basal production of PGE_2 and $PGF_{2α}$ in frozen and unfrozen cells.

RESULTS AND DISCUSSION

Frozen stored buffalo endometrial stromal cells immediately after thawing appeared as single or in small clumps, glistening and rounded which changed their morphology from rounded to flat spindle shaped immediately 24 h after seeding retaining their normal morphological characteristics and reached confluence or monolayer by day 7 culture (Figure 1-A,B,C). The viability of frozen thawed stromal cells at the time of seeding estimated by Trypan blue dye exclusion was 63.3%. The loss of viability may be due to stress of freezing-thawing process causing injury to the plasma membrane, a primary target of cryo-damage (Gao and Crister, 2000). Production of PGE_2 is a useful parameter for determining the functional status of bovine endometrial stromal cells. PGE_2 has been reported as one of the principal prostaglandin secreted by endometrial stromal cells in cattle and buffalo (Parent *et al.,* 2003; Gupta *et al.*, 2010). There was no significant difference in the basal production of PGE_2 and $PGF_{2α}$ by the frozen-thawed and unfrozen buffalo stromal cells (289.38 ± 4.39, 3.28 ± 0.16 pg/µg protein vs. 305.97 ± 3.20, 4.26 ± 0.17 pg/µg protein, respectively) on day 7 of culture indicating no adverse effect of cryopreservation on their functional characteristics. No difference in the production of PGE_2 and $PGF_{2α}$ by frozen thawed stromal cells as compared to unfrozen cells indicated resistance of stromal cells to the freezing and thawing process (Murakami *et al.,* 2003). The results also corroborated with the finding of Szostek *et al.* (2012) in equines. In the present study successful cryopreservation of buffalo endometrial stromal cells without any

Table 1. Basal prostaglandin production (pg/µg protein) by frozen thawed buffalo endometrial stromal cells.

Groups	**PGE_2**	**$PGF_{2\alpha}$**
Frozen stromal cells	289.38 ± 4.39	3.28 ± 0.16
Unfrozen stromal cells (Control)	305.97 ± 3.20	4.26 ± 0.17
‘t’ value	3.05 ^{NS}	4.06 ^{NS}

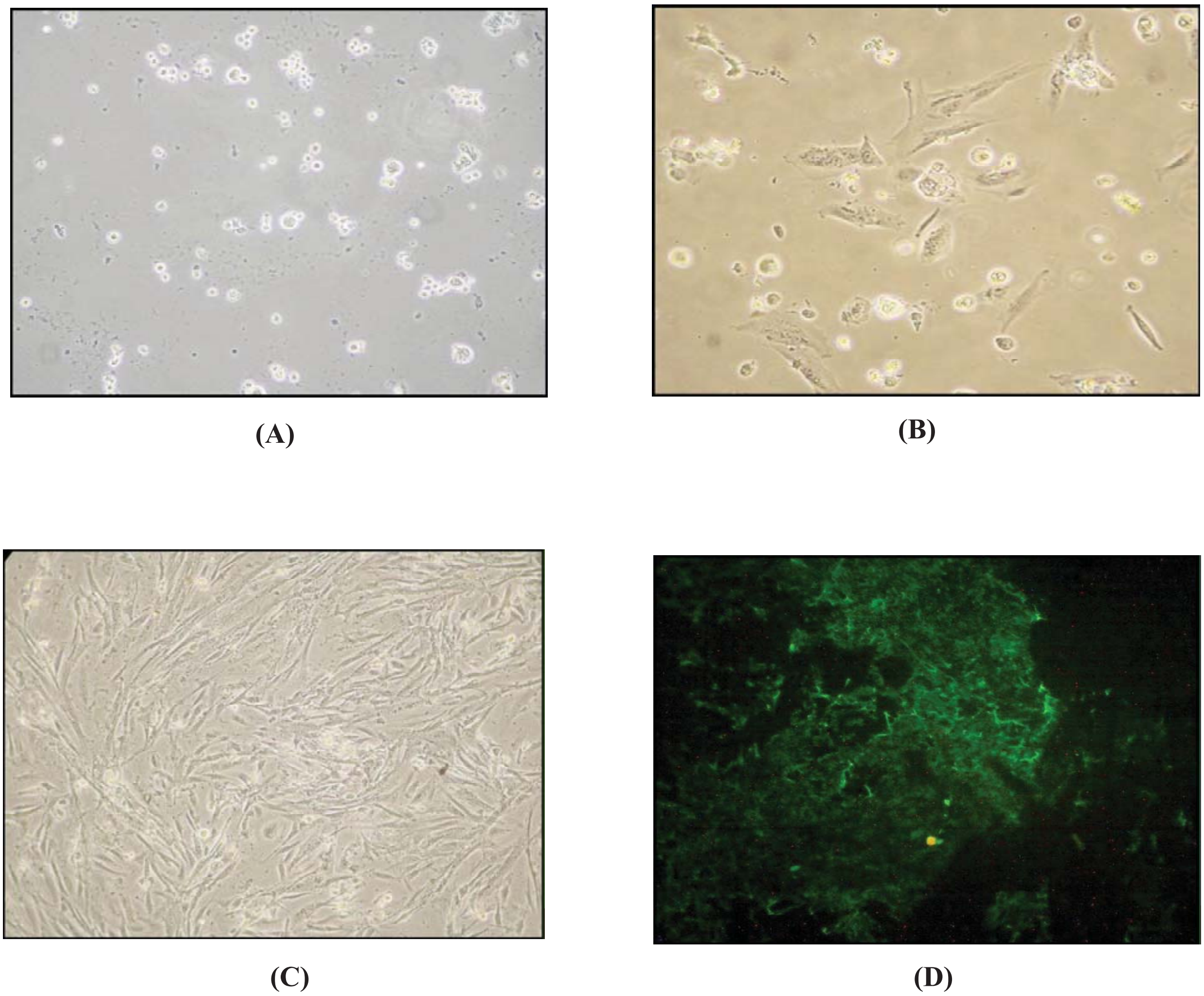

Figure 1. Frozen-thawed buffalo endometrial stromal cell immediately after seeding (A), 24 h after seeding (B), at confluence/ monolayer (C) Immunofluorescent staining of buffalo endometrial stromal cells (stained positive with anti-fibronectin) (D).

major impairment in morphological and functional integrity was achieved and may serve as useful model to study cell biology and physiology of the endometrium particularly in buffalos.

ACKNOWLEDGEMENTS

The project is funded by the National Fund for Basic and Strategic Research in Agricultural Sciences (NFBSRA) under the Indian Council of Agricultural Research, New Delhi, India. The authors thank the Director, IVRI, Izatnagar, Bareilly, UP for necessary facilities to carry out this research work.

REFERENCES

Acosta, T.J., S. Yoshioka, J. Komiyama, S.H. Lee, A.T. Grazul-Bilska and D.J. Skarzynski. 2007. Effects of storage and passage of bovine luteal endothelial cells on endothelin-1 and prostaglandin $F_{2\alpha}$ production. *J. Reprod Dev.,* **53**: 473-480.

Asselin, E., P. Drolet and M.A. Fortier. 1997. Cellular mechanisms involved during oxytocin induced prostaglandin $F_{2\alpha}$ production in endometrial epithelial cells *in vitro:* Role of cyclooxygenase-2. *Endocinology,* **138**(11): 4798-4805.

Bradford, M.M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal. Biochem*., **72**: 248-254.

Fortier, M.A., L.A. Guibault and F. Grasso. 1988. Specific properties of epithelial and stromal cells from the endometrium of cows. *J. Reprod. Fertil*., **83**: 239-248.

Freshney, R.I. 2000. *Culture of Animal Cells: A Manual of Basic Technique*, 4th ed. Wiley-Liss Inc., New York, USA. p. 361.

Gao, D. and J.K. Crister. 2000. Mechanism of cryoinjury in living cells, *ILAR J.,* **41**: 187-196.

Gupta, C., S.K. Singh, R.K. Baithalu, K.R. Anuj, A. Saxena, R.P. Singh and S.K. Agarwal, 2010. Morphological and functional characterization of endometrial stromal cells in buffalo, p. 119. *In International Symposium on "Biotechnologies for Optimization of Reproductive Efficiency of Farm and Companion Animals to Improve Global Food Security & Human Health" and XXVI Annual Convention of ISSAR*, G.B.P.U.A. & T., Pantnagar.

Guzeloglu, A., F. Michel and W.W. Thatcher. 2004. Differential effects of interferon-τ on the prostaglandin synthetic pathway in bovine endometrial cells treated with phorbol ester. *J. Dairy Sci.*, **87**: 2032-2041.

Kasimanickama, K., T.F. Duffielda, R.A. Fosterb, C.J. Gartleya, K.E. Lesliea, J.S. Waltonc and W.H. Johnsona. 2004. Endometrial cytology and ultrasonography for the detection of subclinical endometritis in postpartum dairy cows. *Theriogenology*, **62**: 9-23.

Kim, T.S., E.H. Kwon, S.U. Kim, H.S. Choi, H.J. Lim and S.B. Park. 2010. Activation and expression of urokinase-type plasminogen activator are modulated by freezing/thawing process through activation of redox signal pathway in primary porcine endometrial cells. *Cryobiology,* **60**: 211-216.

Murakami, S., M. Shibaya, K. Takeuchi, D.J. Skarzynski and K. Okudu. 2003. A passage and storage system for isolated bovine endometrial epithelial and stromal cells. *J.*

Reprod Develop., **49**(6): 531-538.

Parent, J., C. Villenenve, A.P. Alexenko, A.D. Ealy and M.A. Fortier. 2003. Influence of different isoforms of recombinant trophoblastic interferons on prostaglandin production in cultured bovine endometrial cells. *Biol. Reprod.,* **68**(3): 1035-1043.

Popov, Y.N. 1969. Diagnosis of occult endometritis in cows (using white side test on cervical mucus). *Veterinariya Moscow*, **4**: 85-87.

Szostek, A.Z., M.J. Siemieniuch, A.M. Galvao, K. Lukasik, D. Zieba, G.M. Ferreira-Dias and Skarzynski. 2012. Effects of cell storage and passage on basal and oxytocin-regulated prostaglandin secretion by equine endometrial epithelial and stromal cells. *Theriogenology*, **77**: 1698-1708.